OXIDATION BY HYDROGEN PEROXIDE

Field of the Invention

The invention relates to a method of carrying out an oxidation reaction.

Background of the Invention

Monooxygenase enzymes catalyse the oxidation of a very wide range of substrates. In order to catalyse the reaction, a monooxygenase enzyme generally requires a cofactor and at least one electron-transfer partner protein (reductase). However, monooxygenase enzymes are capable of using hydrogen peroxide (H_2O_2) as an oxidizing agent because it acts as a source of dioxygen and two electrons. The use of H_2O_2 to drive oxidation reactions is known as the "peroxide shunt".

Summary of the invention

Monooxygenase enzymes generally have a high K_m for H_2O_2, (such as about 20mM) in comparison to peroxidase enzymes. As a result, high concentrations of H_2O_2 are required for appreciable levels of activity of a monooxygenase enzyme when the oxidation reaction is performed using the peroxide shunt. For example, the initial rate of monooxygenase activity using 50mM H_2O_2 is far below that when the natural co-factor, NAD(P)H, is used as with the physiological electron-transfer partners.

The invention provides a more efficient method of carrying out an oxidation reaction using the peroxide shunt by reducing the oxidative damage that occurs to the monooxygenase enzyme by not allowing excess levels of H_2O_2 to be present whilst the reaction is carried out.

Simultaneous production of H_2O_2 at a rate less than or equal to the rate at which it is used in an oxidation reaction catalysed by monooxygenase results in improved efficiency of the oxidation reaction and an increase in the product yield. Various methods may be used to produce H_2O_2 at the required rate, such as use of an electrochemical reaction, an enzyme or a precursor.

Accordingly, the present invention provides a method of carrying out an oxidation reaction catalysed by a monooxygenase enzyme and using hydrogen peroxide as an oxidant, in which reaction a low level of oxidation damage of the monooxygenase occurs, said method comprising producing the hydrogen peroxide

simultaneously with the oxidation reaction, wherein the hydrogen peroxide is produced at a rate less than or equal to the rate at which it is used in the reaction.

The present invention also provides a method of carrying out an oxidation reaction catalysed by a monooxygenase enzyme and using hydrogen peroxide as an oxidant, in which reaction a low level of oxidation damage of the monooxygenase occurs, said method comprising carrying out the reaction in the presence of an H_2O_2 or hydroxyl radical sequestering agent that controls the H_2O_2 or hydroxyl radical concentration.

Description of the Sequences

SEQ ID NO: 1 shows the nucleotide sequence of cytochrome P450Cam from *Pseudomonas putida.*

SEQ ID NO: 2 shows the amino acid sequence of cytochrome P450Cam from *Pseudomonas putida.*

SEQ ID NO: 3 shows the nucleotide sequence of cytochrome P450BM-3 from *Bacillus megaterium.*

SEQ ID NO: 4 shows the amino acid sequence of cytochrome P450 BM-3 from *Bacillus megaterium*. The first 472 amino acid residues form the heme domain. The last 585 amino acid residues form the reductase domain. All 1048 amino acid residues form the holoenzyme.

The convention in the art, which is adopted herein, is to refer to a mutant with reference to the native amino acid residue at a position in the sequence, followed by the amino acid at that position in the mutant, e. g., F87 refers to the phenylalanine at position 87 in the wild-type sequence, and F87A refers to the phenylalanine at position 87 in the wild-type sequence which has been changed to alanine in the variant. The numbering of the amino acid residues starts with the amino acid residue following the initial methionine residue.

Mutants used in Examples were F87A (single mutation; SEQ ID NOs: 5 and 6) and F87V L188Q A74G (triple mutation; SEQ ID NOs: 7 and 8).

SEQ ID NO: 5 shows the amino acid sequence of the F87A mutant of cytochrome P450BM-3 from *Bacillus megaterium.*

SEQ ID NO: 6 shows the nucleotide sequence of of the F87A mutant of cytochrome P450BM-3 from *Bacillus megaterium.*

SEQ ID NO: 7 shows the amino acid sequence of the F87V L188Q A74G

mutant of cytochrome P450BM-3 from *Bacillus megaterium*.

SEQ ID NO: 8 shows the nucleotide sequence of of the F87V L188Q A74G mutant of cytochrome P450BM-3 from *Bacillus megaterium*.

SEQ ID NO: 9 shows the nucleotide sequence of subunit 1 of B-276 alkene epoxidase from *Nocardia coralline*.

SEQ ID NO: 10 shows the amino acid sequence of subunit 1 of B-276 alkene epoxidase from *Nocardia coralline*.

SEQ ID NO: 11 shows the nucleotide sequence of subunit 2 of B-276 alkene epoxidase from *Nocardia coralline*.

SEQ ID NO: 12 shows the amino acid sequence of subunit 2 of B-276 alkene epoxidase from *Nocardia coralline*.

SEQ ID NO: 13 shows the nucleotide sequence of the alpha subunit of Py2 alkene monooxygenase from *Xanthobacta* sp.

SEQ ID NO: 14 shows the amino acid sequence of the alpha subunit of Py2 alkene monooxygenase from *Xanthobacta* sp.

SEQ ID NO: 15 shows the nucleotide sequence of the beta subunit of Py2 alkene monooxygenase from *Xanthobacta* sp.

SEQ ID NO: 16 shows the amino acid sequence of the beta subunit of Py2 alkene monooxygenase from *Xanthobacta* sp.

SEQ ID NO: 17 shows the nucleotide sequence of the gamma subunit of Py2 alkene monooxygenase from *Xanthobacta* sp.

SEQ ID NO: 18 shows the amino acid sequence of the gamma subunit of Py2 alkene monooxygenase from *Xanthobacta* sp.

SEQ ID NO: 19 shows the nucleotide sequence of the alpha subunit of soluble methane monooxygenase from *Methylococcus capsulatas*.

SEQ ID NO: 20 shows the amino acid sequence of the alpha subunit of soluble methane monooxygenase from *Methylococcus capsulatas*.

SEQ ID NO: 21 shows the nucleotide sequence of the beta subunit of soluble methane monooxygenase from *Methylococcus capsulatas*.

SEQ ID NO: 22 shows the amino acid sequence of the beta subunit of soluble methane monooxygenase from *Methylococcus capsulatas*.

SEQ ID NO: 23 shows the nucleotide sequence of the gamma subunit of soluble methane monooxygenase from *Methylococcus capsulatas*.

SEQ ID NO: 24 shows the amino acid sequence of the gamma subunit of

soluble methane monooxygenase from *Methylococcus capsulatas.*

SEQ ID NO: 25 shows the nucleotide sequence of GPo1 alkane hydroxylase (*Alk*B gene) from *Pseudomonas oleovorans.*

SEQ ID NO: 26 shows the amino acid sequence of GPo1 alkane hydroxylase from *Pseudomonas oleovorans.*

SEQ ID NO: 27 shows the nucleotide sequence of the alpha subunit of toluene 2-monooxygenase from *Burkholderia cepacia.*

SEQ ID NO: 28 shows the amino acid sequence of the alpha subunit of toluene 2-monooxygenase from *Burkholderia cepacia.*

SEQ ID NO: 29 shows the nucleotide sequence of the beta subunit of toluene 2-monooxygenase from *Burkholderia cepacia.*

SEQ ID NO: 30 shows the amino acid sequence of the beta subunit of toluene 2-monooxygenase from *Burkholderia cepacia.*

SEQ ID NO: 31 shows the nucleotide sequence of the gamma subunit of toluene 2-monooxygenase from *Burkholderia cepacia.*

SEQ ID NO: 32 shows the amino acid sequence of the gamma subunit of toluene 2-monooxygenase from *Burkholderia cepacia.*

SEQ ID NO: 33 shows the nucleotide sequence of phenol hydroxylase (pheA) gene from *Bacillus stearothermophilus.*

SEQ ID NO: 34 shows the amino acid sequence of phenol hydroxylase gene from *Bacillus stearothermophilus.*

SEQ ID NO: 35 shows the nucleotide sequence of stearoyl-ACP desaturase from *Helianthus annuus.*

SEQ ID NO: 36 shows the amino acid sequence of stearoyl-ACP desaturase from *Helianthus annuus.*

Detailed description of the Invention

It is to be understood that this invention is not limited to particular embodiments. It is also to be understood that different applications of the disclosed methods may be tailored to the specific needs in the art. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

In addition as used in this specification and the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the content clearly

dictates otherwise. Thus, for example, reference to "a substrate" includes two or more substrates, reference to "an enzyme" includes reference to two or more enzymes, and the like.

All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

The methods of the invention enable the oxidation of a variety of substrates. Such substrates include, but are not limited to, alkanes, aromatic compounds, terpenoid compounds, alkenes and fatty acids.

Suitable alkanes include, but are not limited to, methane, ethane, propane, butane, pentane, hexane, heptane, *n*-octane, *n*-nonane, *n*-decane, *n*-dodecane and *n*-hexadecane. The oxidation of alkanes produces alcohols. The oxidation of methane to methanol is technologically and economically very important. The medium-chain alcohols (e.g. *n*-octanol) are synthetic intermediates while the longer chain alcohols (e.g. *n*-dodecanol) are used for the synthesis of fatty acid derivatives.

Suitable aromatic compounds include, but are not limited to, benzene, toluene, xylene, chlorobenzene, phenol and substituents thereof. The phenolic and catecholic products are used in the synthesis of fragrance and flavour compounds.

Suitable terpenoid compounds include, but are not limited to, monoterpenes such as limonene, pinene, terpinene, and ocimene, sesquiterpenes such as valencene and aromadendrene and triterpenes which include the steroidal compounds. The products are intermediates for synthesis, fine fragrance and flavouring chemicals and pharmaceuticals.

Suitable alkenes include, but are not limited to, simple molecules such as propene, hex-1-ene, hex-2-ene, and styrene, and carbon-carbon double bonds in complex molecules. Selective epoxidation of alkenes to a single enantiomer is very important in synthesis. Optically pure propene oxide and styrene oxide are very useful intermediates in synthesis.

Hydroxylated fatty acids are precursors to polymers.

Monooxygenase enzyme

The enzyme used to carry out an oxidation reaction according to the invention is a monooxygenase enzyme. A person skilled in the art can determine whether an enzyme is a monooxygenase enzyme using standard techniques in the art. Typically, the prosthetic groups may be characterised using protein crystallography,

especially for non-heme iron enzymes because they generally do not have chromophores. Otherwise, a person skilled in the art will typically use sequence alignment, looking for conserved motifs such as the active site, and iron content as well as subunit composition.

The monooxygenase enzyme preferably has a K_m for H_2O_2 of at least 15nM, at least 20nM, at least 25nM, at least 30nM, at least 35nM, at least 40nM, at least 45nM or at least 50nM.

Examples of monooxygenase enzymes include, but are not limited to, cytochrome P450 monooxygenases and non-heme di-iron monooxygenase enzymes. Suitable non-heme di-iron monooxygenase enzymes include, but are not limited to methane monooxygenase (Colby *et al.*, Biochem. J., 1977; 165: 395-402; Dalton, Adv. Appl. Microbiol., 1980; 26: 71-87; Fox *et al.*, J. Biol. Chem., 1989; 264: 10023-10033; Fox *et al.*, Methods Enzymol., 1990; 188: 191-202; McDonald *et al.*, Appl. Environ. Microbiol., 1997; 63: 1898-1904), alkane hydroxylase (van Beilen *et al.*, Enzyme Microb. Technol., 1994; 16: 904-911), toluene monooxygenase (Luykx *et al.*, Biochem. Biophys. Res. Commun., 2003; 312: 373-379; Pikus *et al.*, Biochemistry, 1996; 35: 9106-9119; Newman & Wackett, Biochemistry, 1995; 34: 14066-14076), alkene monooxygenase (Gallagher *et al.*, Eur. J. Biochem., 1997; 247: 635-641; Lange & Que, Curr. Opin. Chem. Biol., 1998; 2: 159-172; Zhou *et al.*, FEBS Lett., 1998; 430: 181-185), phenol monooxygenase (Divari *et al.*, Eur. J. Biochem., 2003; 270: 2244-2253) and steroid desaturase (Shanklin *et al.*, Biochemistry, 1994; 33: 12787-12794). The non-heme di-iron monooxygenase enzymes are typically of eukaryotic or prokaryotic origin and preferably of bacterial, fungal, yeast, plant or animal origin. Preferred sequences are shown in SEQ ID NOs: 1 to 36.

The enzyme used in the methods of the invention is preferably a cytochrome P450 enzyme, typically of eukaryotic or prokaryotic origin. Cytochrome P450 monooxygenases are typically characterised by a 446-450 nm heme Soret band for the ferrous-carbon monoxide complex. The enzyme is generally of bacterial, fungal, yeast, plant or animal origin, and thus may be from a bacterium of the genus *Pseudomonas*. The enzyme may be a naturally-occurring form of P450, such as $P450_{cam}$, $P450_{BM-3}$ from *Bacillus megaterium,* $P450_{terp}$ from *Pseudomonas sp,* $P450_{eryF}$ from *Saccharopollyspora erythraea* and also P450 105 D1 (CYP105) from *Streptomyces griseus* strains.

Alternatively, the enzyme may be a mutant of a naturally-occurring form of P450. The mutants retain the essential biological activity of the naturally-occurring enzyme, namely the ability to catalyse an oxidation reaction using H_2O_2. The mutant may have one or more mutations in the active site of the enzyme.

An amino acid 'in the active site' is one which lines or defines the site in which the substrate is bound during catalysis or one which lines or defines a site through which the substrate must pass before reaching the catalytic site. Therefore such an amino acid typically interacts with the substrate during entry to the catalytic site or during catalysis. Such an interaction typically occurs through an electrostatic interaction (between charged or polar groups), hydrophobic interaction, hydrogen bonding or van der Waals forces.

The amino acids in the active site can be identified by routine methods to those skilled in the art. These methods include labelling studies in which the enzyme is allowed to bind a substrate which modifies ('labels') amino acids which contact the substrate. Alternatively the crystal structure of the enzyme with bound substrate can be obtained in order to deduce the amino acids in the active site.

The monooxygenase enzyme may have 1, 2, 3, 4, 5 to 10, 10 to 20 or more other mutations, such as substitutions, insertions or deletions. Amino acid substitutions may be made to the amino acid sequence of a naturally-occurring enzyme, for example from 1, 2, 3, 4 or 5 to 10, 20 or 30 substitutions. Conservative substitutions may be made, for example, according to Table 1. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

Table 1 – Conservative amino acid substitutions

NON-AROMATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar - charged	D E
		H K R
AROMATIC		H F W Y

The mutations may be in the active site or outside the active site. Typically the mutations are in the 'second sphere' residues which affect or contact the position or orientation of one or more of the amino acids in the active site. The insertion is typically at the N and/or C terminal and thus the enzyme may be part of a chimeric protein. The deletion typically comprises the deletion of amino acids which are not involved in catalysis, such as those outside the active site (thus the enzyme is a mutated fragment of a naturally occurring enzyme). The monooxygenase enzyme may thus comprise only those amino acids which are required for oxidation activity.

The mutation in the active site typically alters the position and/or conformation of the substrate when it is bound in the active site. The mutation may make the site on the substrate which is to be oxidized more accessible to the heme group. Thus the mutation may be a substitution to an amino acid which has a smaller or larger, or more or less polar, side chain.

The mutations typically increase the stability of the protein, or make it easier to purify the protein. They typically prevent the dimerisation of the protein, typically by removing cysteine residues from the protein (e.g. by substitution of cysteine at position 334 of $P450_{cam}$, or at an equivalent position in a homologue, preferably to alanine). They typically allow the protein to be prepared in soluble form, for example by the introduction of deletions or a poly-histidine tag, or by mutation of the N-terminal membrane anchoring sequence. The mutations typically inhibit protein oligomerisation, such as oligomerisation arising from contacts between hydrophobic patches on protein surfaces.

The mutations may affect the manner in which the enzyme utilises H_2O_2 and thereby improve the efficiency of the reaction. For example, replacement of all the methionine residues of the heme domain of $P450_{BM-3}$ with norleucine results in a two-fold increase in the peroxygenase activity of the enzyme (Cirino *et al.*, Biotechnol. Bioeng., 2003; 83(6): 729-734). Furthermore, direct evolution studies to find mutants of enzymes more resistant to peroxide have been reported (Cirino & Arnold, Angew. Chem. Int. Ed., 2003; 42: 3299-3301).

Thus the mutant enzyme is typically at least 70% homologous to a naturally occurring enzyme on the basis of amino acid identity.

A mutant protein (i.e. described as being a mutant of another protein) mentioned herein is typically at least 70% homologous to the relevant protein or at least 80 or 90% and more preferably at least 95%, 97% or 99% homologous thereto

over at least 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous amino acids. The contiguous amino acids may include the active site. This homology may alternatively be measured not over contiguous amino acids but over only the amino acids in the active site.

Homology can be measured using known methods. For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology (for example used on its default settings) (Devereux *et al* (1984) *Nucleic Acids Research* **12**, p387-395). The PILEUP and BLAST algorithms can be used to calculate homology or line up sequences (typically on their default settings), for example as described in Altschul S. F. (1993) J Mol Evol 36:290-300; Altschul, S, F *et al* (1990) J Mol Biol 215:403-10.

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul *et al*, supra). These initial neighbourhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) *Proc. Natl. Acad. Sci.* USA 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.* USA 90: 5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences

would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

Mutants include fragments of the above-mentioned sequences. Such fragments retain monooxygenase activity. Fragments may be at least 300, at least 400 or at least 450 amino acids in length. Such fragments may be used to produce chimeric enzymes as described in more detail below.

Mutants also include chimeric proteins comprising fragments or portions of a naturally-occurring enzyme. One or more amino acids may be alternatively or additionally added to the polypeptides described above. An extension may be provided at the N-terminus or C-terminus of the naturally-occurring enzyme or variant or fragment thereof. The extension may be quite short, for example from 1 to 10 amino acids in length. Alternatively, the extension may be longer. A carrier protein may be fused to an amino acid sequence described above. A fusion protein incorporating one of the enzymes described above can thus be used in the invention.

The naturally-occurring enzyme or mutant thereof may also be chemically-modified. A number of side chain modifications are known in the art and may be made to the side chains of the enzymes discussed above. Such modifications include, for example, glycosylation, phosphorylation, modifications of amino acids by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$, amidination with methylacetimidate or acylation with acetic anhydride. The modification is preferably glycosylation.

The mutations discussed herein are generally introduced into the enzyme by using methods known in the art, such as site directed mutagenesis of the enzyme, PCR and gene shuffling methods or by the use of multiple mutagenic oligonucleotides in cycles of site-directed mutagenesis. Thus the mutations may be introduced in a directed or random manner. The mutagenesis method thus produces one or more polynucleotides encoding one or more different mutants. Typically a library of mutant oligonucleotides is produced which can be used to produce a library of mutant enzymes.

The enzyme may be made synthetically or by recombinant means using methods known in the art. The amino acid sequence of the monooxygenase enzyme may be modified to include non-naturally occurring amino acids or to increase the

stability of the enzyme. When the enzyme is produced by synthetic means, such amino acids may be introduced during production. The proteins or peptides may also be modified following either synthetic or recombinant production.

The enzyme may also be produced using D-amino acids. In such cases the amino acids will be linked in reverse sequence in the C to N orientation. This is conventional in the art for producing such proteins or peptides.

The enzyme may be produced in a cell by *in situ* expression of the polypeptide from a recombinant expression vector. The expression vector optionally carries an inducible promoter to control the expression of the polypeptide. The enzyme may be produced in large scale following purification by any protein liquid chromatography system after recombinant expression. Preferred protein liquid chromatography systems include FPLC, AKTA systems, the Bio-Cad system, the Bio-Rad BioLogic system and the Gilson HPLC system.

Oxidation reaction

The methods of the invention concerns carrying out a high efficiency oxidation reaction catalysed by a monooxygenase enzyme. A high efficiency oxidation reaction is a reaction that occurs without an appreciable reduction in the enzyme turnover or product yield or inactivation of the monooxygenase enzyme. Preferably, the monooxygenase enzyme displays at least 70%, at least 80%, at least 90%, at least 95% or 100% of the activity shown at the beginning of the reaction after 1 hour, 2 hours, 6 hours, 12 hours, 1 day, 2 days or 5 days.

Typically the methods of the invention are carried out in vitro, such as in a cell free system.

The reaction is driven by the "peroxide shunt". The reaction of the invention is carried out in the presence of the monooxygenase enzyme (a), the substrate (b) and H_2O_2 (c). The reaction is typically performed in aerobic conditions and does not require any cofactors. The production of (c) is discussed in more detail below. In this system the flow of electrons is typically: (c) → (a) → (b).

In the methods the concentration of (a) and (b) is typically from 10^{-8} to 10^{-2}M, preferably from 10^{-6} to 10^{-4}M. Typically the ratio of concentrations of (a): (b) is from 0.1:10 to 1:10, preferably from 1:0.5 to 1:2, or from 1:0.8 to 1:1.2. Preferably, the concentration of (b) is greater than the concentration of (a). The preferred concentration of (a) is that which when reacted with substrate will generate sufficient

product to be detected by available analytical methods e.g. GC, HPLC. This is typically of the order of μM quantities.

Generally the methods are carried out at a temperature and/or pH at which the monooxygenase enzyme is functional, such as when the enzyme has at least 20%, 50%, 80% or more of peak activity. Typically the pH is from 2 to 11, such as from 5 to 9 or from 6 to 8, preferably from 7 to 7.8 or 7.4. The pH can be maintained using a suitable buffering agent such as phosphate or acetate based systems. Typically the temperature is from 0 to 80°C, such as from 25 to 75°C, from 30 to 60°C or from 50°C to 80°C. Preferably, the temperature is from 20 to 40°C.

Typically in the methods at least 20 turnovers/min occur, such as at least 50, 100, 200, 300, 500 or more turnovers (turnover is measured as nanomoles of product formed per nanomole of enzyme).

Typically, the rate of H_2O_2 production is less than or equal to 1, 2 or 3 μg per min per mg of monooxygenase enzyme. Typically, the concentration of H_2O_2 throughout the reaction is less than or equal to 0.1, 0.5 or 1mM. Typically, the reaction continues for at least 60 minutes, at least 240 minutes, at least 6 hours or at least 10 hours.

The methods of the invention may be carried out in the monooxygenase substrate if it is a liquid under the reaction conditions. The methods of the invention may also be conducted in a solvent. Suitable solvents include, but are not limited to, water, aqueous buffer solutions mixed water/organic and aqueous buffer/organic solvent systems. Preferably, the organic solvent is a hydrocarbon such as hexane, benzene, acetonitrile, lower aliphatic alcohols, ketones and dioxane, dimethylformamide and dimethylsulphoxide and mixtures thereof. The solvent is typically one in which the reagants and products are highly soluble and one that maintains the stability and activity of the monooxygenase enzyme.

The reaction may be carried out in a homogenous system with all the components in solution. Typically, the monooxygenase enzyme and substrate are mixed together in a suitable solvent in a stirred tank reactor and the reaction is conducted in batch, semi-batch or continuous mode.

Alternatively, the monooxygenase enzyme may be immobilized on a suitable solid support, such as silica, prior to carrying out the method of the invention. An immobilized monooxygenase enzyme can be packed into a fixed bed

reactor and the substrate passed over the enzyme. In one embodiment, the enzyme producing the H_2O_2 (discussed in more detail below) may be immobilized on the same or different material as the monooxygenase enzyme. Procedures for immobilizing enzymes are known in the art. Examples of such procedures include, but are not limited to, covalent coupling to insoluble organic or inorganic supports, entrapment in gels and adsorption to ion exchange resins or other adsorbent materials. (G. F. Bickerstaff ed., "Immobilization of Enzymes and Cells," Humana Press, Totowa. New Jersey, 1997).

In a further embodiment, a membrane on the "entry" side admits the substrate slowly from the "reactant" side and then a hydrophilic membrane on the "exit" side allows hydrophilic compounds to flow out to the "product" side of the flow reaction cell. In this case the H_2O_2 may be generated outside the membrane and allowed to flow through the membrane to the mobile or immobile enzyme.

In one embodiment, H_2O_2 is preferably produced by one of the methods discussed in more detail below. In another embodiment, a H_2O_2 or hydroxyl radical sequestering agent is used to sequester excess H_2O_2 or hydroxyl radical during the oxidation reaction. The sequestering agent may be a chelating agent. In one embodiment, the chelating agent is EDTA. The EDTA inhibits production of the hydroxyl radical, for example, produced by the reaction of trace amounts of iron (or copper) with the H_2O_2.

H_2O_2 production by an electrochemical reaction

The H_2O_2 may be produced in the method of the invention by an electrochemical reaction. An electrochemical reaction is generally a means for introducing a current to a liquid, preferably a solution. An electrochemical reaction is typically an oxidation or reduction reaction that takes place at an electrode through which a current flows. An electrode is a solid capable of conducting electricity, typically carbon-based or metallic, leading to an external source or sink which is in contact with the liquid, preferably a solution. The electrode may be either positively charged (cathode) or negatively charged (anode). Two or more electrodes may form an electrochemical cell from which an external wire can lead from each electrode to an external electrical device. An oxidation or reduction reaction takes place at one electrode, while a redox reaction can take place either in an electrochemical cell or directly in the liquid.

Production of H_2O_2 using an electrochemical reaction is energy efficient. H_2O_2 is typically produced by the controlled electrochemical reduction of molecular oxygen to hydrogen peroxide. The surface area and the overpotential of the cathode are key considerations for the two-electron reduction of molecular oxygen to hydrogen peroxide. Typically, carbon-based cathodes are used and they may be modified with a compound known to lower the overpotential for this reaction. Electrode materials and modifiers which will perform this task effectively and efficiently are well known in the art. The reduction of O_2, and hence production of hydrogen peroxide, can typically be controlled by the potential applied to the cathode. The potential applied to the cathode will vary depending on the cathode and any modifications to the cathode made.

The electrochemical reaction used in the method of the invention may be the sonoelectrochemical reduction of dioxygen. This method is well known in the art (Compton *et al.*, Electroanalysis, 1997; 9(7): 509-522).

H_2O_2 production by an enzyme

The H_2O_2 may be produced in the method of the invention by an enzyme. The enzyme is preferably an oxidase. Examples of suitable oxidases include, but are not limited to, glucose oxidase (E.C. 1.1.3.4), secondary-alcohol oxidase (E.C. 1.1.3.18), methanol oxidase (E.C. 1.1.3.31), oxalate oxidase (E.C. 1.2.3.4), aryl-aldehyde oxidase (E.C. 1.2.3.9), carbon monoxide oxidase (E.C. 1.2.3.10), amine oxidase (E.C. 1.4.3.4), ethanolamine oxidase (E.C. 1.4.3.8), nitroethane oxidase (E.C. 1.7.3.1) and sulfite oxidase (E.C. 1.8.3.1). Glucose oxidase (E.C. 1.1.3.4) catalyzes the conversion of D-glucose to D-glucono-1,5-lactone and H_2O_2.

Secondary-alcohol oxidase (E.C. 1.1.3.18) catalyzes the conversion of a secondary alcohol to a ketone and H_2O_2. Methanol oxidase (E.C. 1.1.3.31) catalyzes the conversion of methanol to formaldehyde and H_2O_2. Oxalate oxidase (E.C. 1.2.3.4) catalyzes the conversion of oxalate to carbon dioxide and H_2O_2. Aryl-aldehyde oxidase (E.C. 1.2.3.9) catalyzes the conversion of an aromatic aldehyde to an aromatic acid and H_2O_2. Carbon monoxide oxidase (E.C. 1.2.3.10) catalyzes the conversion of CO and H_2O to carbon dioxide and H_2O_2. Amine oxidase (E.C. 1.4.3.4) catalyzes the conversion of RCH_2NH_2 and H_2O to RCHO and NH_3 and H_2O_2. Ethanolamine oxidase (E.C. 1.4.3.8) catalyzes the conversion of ethanolamine and H_2O to glycolaldehyde and H_2O_2. Nitroethane oxidase (E.C. 1.7.3.1) catalyzes

the conversion of nitroethane and H_2O to acetaldehyde and H_2O_2. Sulfite oxidase (E.C. 1.8.3.1) catalyzes the conversion of sulfite and H_2O_2 to sulfate and H_2O_2. The oxidase may be purchased commercially (e.g., glucose oxidase). Alterantively, the oxidase can be extracted from known microorganisms using procedures known in the art.

The substrate for the oxidase will be well known in the art. In addition to the substrate, the reaction to produce H_2O_2 will also involve water. Typically, a H_2O_2-activating metal is also included in the reaction. Suitable metals include, but are not limited to, cerium, chromium, cobalt, copper, iron, manganese, molybdenum, silver, titanium, tungsten, vanadium and mixtures thereof. Metallosilicates containing the above metals can be prepared and used in the method of the invention. The procedure for producing such metallosilicates in known in the art (Neumann *et al.,* Journal of Catalysis, 1997; 166: 206-127). The metallosilicate is preferably tetrahedrally coordinated titanium such as silicalite-l (TS-1), silicalite-2 (TS-2), zeolite-beta, silicon analogs of ZSM-48 and MCM-4 1. (Murugavel and Roesky, Angew. Chem. Int. Ed. Engl., 1997; 36(5): 477-479).

In a preferred embodiment of the invention, the metal-containing solid or metallosilicate is used as a support upon which the H_2O_2-producing enzyme is immobilized. In another preferred embodiment, the monooxygenase enzyme is also immobilized on the same or different metallosilicate.support.

Preferably, the oxidase is first mixed with the other reaction components and then the reaction is initiated by addition of the oxidase substrate. For example, the monooxygenase enzyme, monoxygenase enzyme substrate and oxidase are all mixed and then the oxidase enzyme is added. In a preferred embodiment, $P450_{BM3}$, octane and glucose oxidase are mixed together and then glucose added. Control of H_2O_2 generation can typically be accomplished by controlling the rate at which the oxidase substrate is added.

H_2O_2 production by a precursor

The H_2O_2 may be produced in the method of the invention by a precursor. The generation of H_2O_2 by the addition of a precursor to water is well known in the art. Precursors include, but are not limited to, salts of perborate, salts of percarbonate, salts of perphosphate and peroxynitrite. Preferred precursors are sodium salts. The H_2O_2-producing properties of the precursor may be enhanced by

using a compound such as tetraacetylethylenediamine. The amount of precursor added to the solution containing the monoxygenase enzyme and substrate is such to maximise the enzymatic reaction with the substrate and to minimise the deactivation of the enzyme by H_2O_2. Preferably the concentration of H_2O_2 produced does not exceed the K_m value for the enzyme but is sufficient to generate the enzyme reactive species.

Examples

Example 1

In this experiment, octane was reacted with electrochemically generated H_2O_2 in the presence of $P450_{BM3}$ heme domain. The experiment was performed at room temperature with a three-electrode configuration in a 100 mL glass beaker. The reticulated vitreous carbon (RVC) cathode, platinum gauze anode and Ag/AgCl reference electrode were contained in the one vessel. The RVC cathode was briefly immersed in a 1 mM 2-aminoanthraquinone ethanolic solution before being removed and allowed to dry in air. The reaction solution contained aqueous Tris buffer (50 mM, pH 7.4) saturated with oxygen, 0.2 M KCl, 0.5 mM octane, and 3 μM $P450_{BM3}$ F87V L188Q A74G heme domain. The reaction solution was stirred to equilibrate (5-10 minutes) and then a potential of –0.55 V vs Ag/AgCl was applied for 2 hours and the solution stirred continuously throughout. GC analysis revealed the presence of the solvent chloroform, octane, 2-, 3- and 4-octanol and the internal standard 1-nonanol. The relative proportion of 2, 3 & 4-octanol was 1:1.1:0.7. The total concentration of octanols formed was 141 μM, representing a turnover per enzyme of 47.

A similar experiment was performed with 1.43 μM wild-type $P450_{BM3}$ heme domain. The total concentration of octanols formed was 8.4 μM, representing a turnover per enzyme of 6. The relative proportion of 2, 3 & 4-octanol in this case was 1:1.7:2.0.

Example 2

In this experiment, octane was reacted with enzymatically generated H_2O_2 in the presence of $P450_{BM3}$ holoenzyme. Into a glass vial was added a solution (total

volume 5 mL) consisting of aqueous Tris buffer (50 mM, pH 7.4), 0.5 mM octane, 1.6 μM $P450_{BM3}$ F87V L188Q A74G holoenzyme and glucose oxidase (1.5 U). After equilibration (5 mins), the reaction was initiated by addition of glucose (1×10^{-6} moles). Successive additions of glucose (1×10^{-6} moles) were made every 5 minutes up to 1 hour (total of 12 additions equivalent to 1.2×10^{-5} moles). The reaction was stirred continuously during this time and stopped after 1.5 hours. GC analysis revealed the presence of the solvent chloroform, octane, 2-, 3- and 4-octanol and the internal standard 1-nonanol. The relative proportion of 2, 3 & 4-octanol was 1:1.1:0.8. The total concentration of octanols formed was 17 μM, representing a turnover per enzyme of 10.

Example 3

In this experiment, octane was reacted with H_2O_2 derived from sodium perborate, in the presence of $P450_{BM3}$ holoenzyme. Into a glass vial was added a solution (total volume 5 mL) consisting of aqueous Tris buffer (40 mM, pH 7.4), 0.5 mM octane, and 1.3 μM $P450_{BM3}$ F87V L188Q A74G holoenzyme. After equilibration (5 mins), the reaction was initiated by addition of $NaBO_3.4H_2O$ (1×10^{-4} moles) and stirred continuously for 1 hour. GC analysis revealed the presence of the solvent chloroform, octane, 2-, 3- and 4-octanol and the internal standard 1-nonanol. The relative proportion of 2, 3 & 4-octanol was 1:1.8:1.1. The total concentration of octanols formed was 77 μM, representing a turnover per enzyme of 59.

For Examples 1 to 3, no octanol products were observed when the P450 enzyme was absent from the solution.

Example 4

In this experiment, pinene was reacted with H_2O_2 derived from sodium perborate, in the presence of $P450_{BM3}$ heme domain. Into a glass vial was added a solution (total volume 5 mL) consisting of aqueous Tris buffer (40 mM, pH 7.4), 0.63 mM pinene, and 3.7 μM wild-type $P450_{BM3}$ heme domain. After equilibration (5 mins), the reaction was initiated by addition of 7.8×10^{-6} moles $NaBO_3.4H_2O$ and stirred continuously for 1 hour. GC analysis revealed the presence of *cis*/*trans* 2,3-epoxides (32%), (+)-*trans*-verbenol (16%), (+)-*cis*-verbenol (6%), (+)-verbenone/(+)-myrtenol (13%), myrtenal (4%), as well as unidentified further

oxidation products (29%). The total concentration of products formed was 80 μM, representing a turnover per enzyme of 22.

Example 5

In this experiment, phenol monooxygenase is reacted with phenol in the presence of with H_2O_2 generated by sodium perborate. Into a glass vial is added a solution (total volume 5 mL) consisting of aqueous Tris buffer (40 mM, pH 7.4), 0.63 mM phenol, and 3.7 μM wild-type phenol monooxygenase. After equilibration (5 mins), the reaction is initiated by addition of 7.8×10^{-6} moles $NaBO_3.4H_2O$ and stirred continuously for 1 hour. GC analysis reveals the presence of oxidation products.

Example 6

In this experiment, $P450_{BM3}$ is reacted with palmitic acid in the presence of H_2O_2 generated by glucose oxidase. Into a glass vial is added a solution (total volume 5 mL) consisting of aqueous Tris buffer (50 mM, pH 7.4), 0.5 mM palmitic acid, 1.6 μM $P450_{BM3}$ holoenzyme and glucose oxidase (1.5 U). After equilibration (5 mins), the reaction is initiated by addition of glucose (1×10^{-6} moles). Successive additions of glucose (1×10^{-6} moles) are made every 5 minutes up to 1 hour (total of 12 additions equivalent to 1.2×10^{-5} moles). The reaction is stirred continuously during this time and stopped after 1.5 hours. GC analysis reveals the presence of oxidation products.

Example 7

Plant CYP74C is reacted with 13 S-hydroperoxylinolenic acid to form the compound 3Z-hexenal (a fragrance). The H_2O_2 is generated by sodium perborate. Into a glass vial is added a solution (total volume 5 mL) consisting of aqueous Tris buffer (40 mM, pH 7.4), 0.63 mM 13 S-hydroperoxylinolenic acid, and 3.7 μM wild-type plant CYP74C. After equilibration (5 mins), the reaction is initiated by addition of 7.8×10^{-6} moles $NaBO_3.4H_2O$ and stirred continuously for 1 hour. GC analysis reveals the presence of oxidation products.